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Faculdade de Farmácia

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Mestrado Integrado em Ciências Farmacêuticas

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Resumo

A Esclerose Múltipla (EM) é uma doença desmielinizante do Sistema Nervoso Central mediada pelo sistema imunitário e é caracterizada por um quadro clínico variável e fisiopatologia heterogénea. Pensa-se que a neurodegeneração presente nesta doença resulta da soma de vários mecanismos biológicos, mas os dados alcançados na literatura confirmam o papel crucial da neuroinflamação (causada por mediadores pro-inflamatórios) e da hipoperfusão cerebral (devido à produção elevada de Endotelina-1). A Sinvastatina e a Fluoxetina são dois exemplos de fármacos em que a experiência do seu uso e estudos indiciam o efeito positivo em doentes com EM. O objetivo deste estudo é verificar se as propriedades neuroprotetoras atribuídas à Sinvastatina e a Fluoxetina na EM envolvem a inibição de Endotelina-1. Para tal será aferido o poder inibidor da Sinvastatina e da Fluoxetina na expressão das citoquinas proinflamatórias TNFα e IL-1β, conhecidas por desencadearem um mecanismo de *feedback* positivo na produção de Endotelina-1 nos tecidos cerebrais.

Recorreu-se a estudos *in vitro* em células de Astrocitoma Humano (1321N1) e de Micróglia de ratinho (BV2) sob *stress* induzido por escassez de nutrientes. Culturas celulares, em placas de 12 ou 24 poços, com diferentes passagens e densidades celulares, foram estimuladas com diferentes concentrações de Sinvastatina (1, 5 e 25 μM) e Fluoxetina (1, 5 e 10 μM) durante 6 ou 24 horas. De seguida as células foram lisadas, recolhidas e a produção de TNFα e IL-1β posteriormente quantificada através de ELISAs. As interferências com falsos positivos de origem desconhecida dificultaram a aferição das citoquinas proinflamatórias, e, parcialmente, os estudos na linhagem celular de Astrocitoma Humano. Nas linhas celulares de microglia de ratinho os resultados obtidos indicam que poderá existir uma correlação negativa entre a concentração de Fluoxetina e a produção de TNFα (p<0,01) e também uma correlação negativa entre a concentração de Sinvastatina e a produção de TNFα e IL-1β (p<0,01). Consequentemente, tal significa que é possível que a Fluoxetina e a Sinvastatina desempenhem um papel neuroprotetor e provavelmente como inibidores indiretos da produção de Endotelina-1. Esta hipótese revela-se promissora mas deverá ser confirmada através de estudos corroborativos assim como a relevância destes resultados para o Homem.

Palavras-chave: Esclerose Múltipla, Neuroinflamação, Citoquinas, Fluoxetina, Sinvastatina.

Abstract

Multiple Sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) that is characterized by a variable clinical course and heterogeneous pathology. The neurodegeneration present in this disease is thought to be the result of multiple pathological pathways, but the collected scientific data undeniably confirms the crucial role of neuroinflammation (caused by proinflammatory mediators) and cerebral hypoperfusion (due to high levels of produced Endothelin-1). Simvastatin and Fluoxetine are two drugs that are being studied due to their possible neuroprotective role and both have demonstrated (through studies and clinical usage) an ameliorative effect in patients with MS. This work's objective is to verify if the neuroprotective properties attributed to Simvastatin and Fluoxetine in MS involve the inhibition of Endothelin-1. To do so, it will be assessed the inhibitor power of Simvastatin and Fluoxetine in the expression of proinflammatory cytokines TNFα and IL-1β, both known for unfold a positive feedback mechanism in the production of Endothelin-1 in brain tissue.

This experiment resorted to *in vitro* studies of human astrocytoma cells (1321N1) and mouse microglia cells (BV2) under starvation induced stress. The cell cultures were grown in 12- or 24- well plates with different passages and cellular densities, stimulated with different concentrations of Simvastatin (1, 5 e 25 μM) or Fluoxetine (1, 5 e 10 μM) for 6 or 24 hours. Next, the cells were lysed, collected and the production of TNFα and IL-1β was later quantified through ELISAs. The interferences with false positives of unknown origin made it difficult to evaluate the levels of produced proinflammatory cytokines, and partially compromised the results obtained from the 1321N1 cell line. The results obtained from the mouse microglia cell line suggest that there might exist a negative correlation between the Fluoxetine concentration and the production of TNFα ($p<0,01$) and also a negative correlation between the concentration of Simvastatin and the production of TNFα and IL-1β ($p<0,01$). Consequentially, this means that there is a possibility that Fluoxetine and Simvastatin may play a neuroprotective role as indirect Endothelin-1 production inhibitors. This hypothesis looks promising, but it should be confirmed through corroborative studies and prove its relevance to Human clinical treatment.

Keywords: Multiple Sclerosis, Neuroinflammation, Cytokines, Fluoxetine, Simvastatin.

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Abreviaturas

- CBF – Cerebral blood flow
- CNS – Central Nervous System
- DMT – Disease Modifying Treatments
- EAE – Experimental Autoimmune Encephalomyelitis
- ELISA – Enzyme-linked immunosorbent assay
- Et-1 – Endothelin-1
- Fluox - Fluoxetine
- IF- γ – Interferon-γ
- IL – Interleukin
- MBC – Myelin basic protein
- MHC – Major Histocompatibility Complex
- MS – Multiple Sclerosis
- NAWM – Normal-appearing white matter
- NF-κB – Nuclear factor kappa-light-chain-enhancer
- NO – Nitric Oxide
- PMS – Progressive MS
- PPMS – Primary Progressive MS
- RRMS – Relapsing Remittent MS
- Simv – Simvastatin
- SPMS – Secondary Progressive MS
- SSRI – Selective Serotonin Reuptake Inhibitor
- TNF – Tumour Necrosis Factor

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1 Introduction

In a world where there is a tangible increase in the number of patients diagnosed with mental and brain illnesses such as Depression, Multiple Sclerosis (MS) or Alzheimer's Disease, it has never been so important to find new ways of studying and approaching these ailments. Together, these diseases are responsible for the majority of the cases of disability in mid-to-later life, and represent a large slice of the global healthcare costs. (1) (2) (3)

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory and demyelinating disease that manifests pathologically and clinically after the self-induced disruption of the dynamic equilibrium of the brain, developing a chronic process that affects the central nervous system's (CNS) integrity and function. During this process occurs the formation of both focal lesions created by acute inflammation, and progressive diffuse axonal degeneration. (4) MS derives from a complex multifactorial etiological process where genetic and environmental agents take a decisive role, often leading to a chronic neurologic disability starting in early to middle adult life. Once added to a wide range of comorbidities, MS represents a real obstacle to the individual's quality of life. (5)

As researchers dwell deeper into the study MS and its epidemiology, it gets clear that it may be influenced by genetic, environmental and epigenetic factors, the latter mostly unidentified. Animal models and studies also suggest that external and internal stress can activate innate inflammatory immune responses, and trigger the ability of immune system cells to produce pro-inflammatory messengers, leading to a wide variety of unwanted symptoms. (6) After being conducted several studies worldwide, it was originally established that the risk of developing MS increased, proportionally with the latitude of said Country, and presented a higher incidence in females. (5)

Prevalence levels were the highest in countries further away from the Equator such as Scandinavian Countries, Canada, Australia or New Zealand. Nonetheless, in the past years the number of new cases in low-risk groups has increased. This fact has been generally attributed to genetic dissemination from high- to low-risk groups, as history favoured racial intermixing in some areas. (7) However, the genetic factors shouldn't be the only ones taken into account when defining risks or causes of disease.(8)

New studies suggest that there might be new environmental triggers worthy of taking into consideration, given the increase of cases reported (besides the technical developments that allow for better diagnosis and healthcare access). (5) (9)

MS has a wide range of symptoms and patterns in which it can express itself in time. Initially, after the occurrence of a first isolated clinical episode with patient-reported symptoms indicating inflammatory demyelination that could be MS, but that has yet to fulfil the needed criteria, the patient is diagnosed with Clinically Isolated Syndrome (CIS). (10)

When diagnosed, approximately 85% of the patients present a relapsing-remitting MS (RRMS), which can be characterized by alternating episodes of neurological impairment (relapses or attacks) followed by complete or partial recovery of symptoms (11). During these episodes of inflammation, patients experience the relapses over the course of several days, becoming maximal after one to two weeks.(12) The symptoms then gradually diminish over the next weeks or months. Nearly two thirds of these patients diagnosed with RRMS will then make a transition to Secondary Progressive MS (SPMS) within two to three decades from the onset. (13) Only about 10% of patients with MS manifest Primary Progressive MS (PPMS) at diagnosis, later showing gradual worsening of neurological disability from symptom incipience with no distinct relapses or remissions. By combining the population affected by SPMS or PPMS, it was created the term – Progressive MS (PMS) (14)

1.2 Physiopathology of MS

The physiopathology of MS isn't fully understood yet, but neuroinflammation is an undeniable factor. Neuroinflammation is characterized by: 1) an initial case of inflammation, believed to be the main trigger of the biological processes leading to CNS tissue damage; 2) demyelination or oligodendrocyte destruction by said inflammation, 3) axonal loss and damage, 4) the presence of activated microglia (glial cells that function as macrophages in the CNS) and gliosis (increased proliferation of glial cells as a response to a harmful stimulus) with or without components of the adaptive immune system (B and T lymphocytes) and 5) increased production of inflammatory mediators, such as cytokines (15) (16) Among these inflammatory mediators are the interferon-γ (IF- γ), Interleukin (IL) 1, IL-1β, Tumour Necrosis Factor (TNF) α and IL-6.

In a normally functioning brain, cytokines' usual low levels are regulated and adjusted so that the appropriate immune response is achieved. However, pro-inflammatory

cytokines may be produced by damaged cells, activated microglia, astrocytes, neurons or activated lymphocytes, and may play an important role as modulators or triggers of astrogliosis. (4) (10) They are also important biomarkers of inflammation, proving themselves very useful in the characterization of MS.(17) Cytokines are soluble proteins and glycoproteins that are usually expressed in small amounts as a response to local specific stimulation.(18) Several studies state that IF-*γ*, TNFα and IL-6 play an important role in MS pathogenesis (4) (5) (19) TNFα has been found in high concentrations within MS lesions, also correlating its presence with the progression of the disease, while IL-6 appears to be directly involved with the neuroinflammation process. Previously, IL-6 was believed to mediate the generation on Th1 cells and modulate the balance between Th1/Th2.(15) In the past decade, it has been proved that IL-6 together with IL-23 are crucial factors for the expansion of a pathogenic CD4+ T cell population to IL-17 producing Th17 cells.(19) This finding is especially relevant since IL-17 presents high concentrations in the active MS brain lesions. Also, Th17 cells have shown to migrate efficiently through the Brain Blood Barrier (BBB) into vulnerable affected areas where they secrete neuron-killing enzymes, such as granzyme B. (20) (13)

As previously stated, several studies and some animal models have indicated that myelin specific CD4+ Th1 and Th17 cells are the driving force in the autoimmune processes in MS. However, there are other cell types that contribute to the pathogenesis of MS, such as CD8+ T cells, NK cells or B cells.(5) It is important to understand that the transition from physiological to pathological autoimmunity involves the loss of immune homeostasis, usually maintained by apoptosis and cell regulatory mechanisms, and also the engagement and activation of lymphocytes proper signalling. These signals can come from either exogenous pathogens such as bacteria or viruses, or from internal homologous structures between endogenous proteins and a pathogen (through a process called molecular mimicry) (5)

1.2.1 Proinflammatory and destruction pathways in MS

To further understand the relevance of the cell types responsible for the neuroinflammation, it is important to know the complex mechanism that leads to the secretion of inflammatory cytokines. The initial step in immune cell activation is to activate dendritic cells via toll like receptors. After activated, they will produce type 1 Interferons. On the other hand, CD4+ T cells are activated once they interact with its specific antigen, presented by the MHC complex of the antigen presenting cell (APC). The CD4+ cell then interacts with the dendritic cell through the T cell Receptor on the

T cell. This induces the activation of CD40 ligand on the surface of T cell, that bind itself to the CD40 receptor on the dendritic cell. This initiates a series of interactions between the two cells, leading to the production of important cytokines from the dendritic cell. Consequentially, the T cell secrete different cytokines such as IL-1, IL-4, IL-10 and IL-12. The presence of all these cytokines later promotes the differentiation into different kinds of Th cells. The resulting cell population will then move out to the site of the inflammation in order to deal with the attacking agent (or endogenous target, in case of autoimmune diseases). (21) (22)

It is believed that the tissue damage seen in the lesions of the CNS of MS patients is caused by an autoreactive CD4+ T-cell-mediated process that facilitates the activation of macrophages/microglia, B cells and cytotoxic CD8+ T cells. These cells may cause damage to the CNS in various ways, recurring to different processes such as cell-, cytokine, antibodies and radical-mediated mechanisms. (15) (23)

Activated microglia cells have an important role in the CNS lesion development, as they produce a wide variety of proteolytic and lipolytic enzymes, nitric oxide (NO) cytotoxic cytokines, toxins and reactive oxygen species. These toxic molecules are potential inducers of axonal injury, specially the oxygen and NO radicals. (24)

Both CD4+ and CD8+ T cells can express ligands for death receptors, being an important factor in MS lesion development. Some of these ligands are the TNF-related apoptosis inducing ligand (TRAIL), Fas ligand and the TNFα. This immunologic attack carried through by the activated T-cells, targets myelin basic proteins (MBP) via the Major Histocompatibility Complex (MHC) molecules presented by antigen-presenting cells. (25) CD8+ T cells are more prevalent in the MS lesions than the CD4+ T cells, as they can recognize antigens present on Human Leukocyte Antigen (HLA) class 1 proteins on the targeted cells. (26) The primary targets are the myelin sheaths and oligodendrocytes, but axons, nerve cells and astrocytes are also affected. (27)

B cell derived plasmatic cells and antibodies have been found in the CNS of MS patients. Their targets however, are still largely unknown, but it is suggested that the primary targets are the proteins of the myelin sheath such as MBP, myelin oligodendrocytes glycoprotein (MOG) and pepteolipid protein (PLP).(28)

1.2.2 Brain Lesions in MS

MS often leads to long-term disability, mainly because of irreversible axonal loss, proven to partially begin in the early stages of the disease as observed in high-resolution histopathological studies. (29) (30) As the acute lesions evolve into a

chronic setting, there is a significant loss of myelin (almost complete demyelination), less mononuclear cells and intense astrogliosis, leading to the formation of multiple of cortical “plaques” (or Sclerotic Patches): isolated scar tissues that are important contributors to motor, sensory and cognitive impairments, and also for giving this disease its name. (5)

These lesions observed in the CNS of MS patients are typically characterized by a demyelinated core separated by a sharp border from normally functional surrounding tissue. These lesions have a higher incidence around the ventricles on the brain white matter, but can also occur further away from the ventricles and on the grey matter.(31)

PMS patients usually don’t show new inflammatory demyelinating plaques, however, there is a predominance of diffuse atrophy of the grey and white matters and changes in the normal-appearing white matter (NAWM) also known as the non-lesion containing tissue. (31)

These changes in the NAWM consist of a diffuse neuroinflammation state, as inflammatory agents are dispersed in the perivascular space across the brain tissue. This inflammatory state is associated with microglia activation, and therefore with diffuse axonal loss.(32) The causes of this diffuse inflammatory state are still unknown, but some research suggests that free-radical induced damage cause my abnormal mitochondrial activity and regulation could be an important factor for the aggravation of MS. (31)

Most lesions found in the grey matter are located in the cerebral cortex. These have a substantially different set of histopathological characteristics from the white matter lesions, and they affect about 80% of the PPMS patients. (33) These lesions have been categorized in three types. They may appear in continuity with subcortical white matter plaques (type 1) or as small intracortical perivascular lesions (type 2). The third and most common type of lesion manifests as a subpial demyelination, appearing as large band-like lesions that extend from the outer surface of the cortex into its deeper layer. (23) (34) Unlike white matter lesions, grey matter lesions don’t show any abnormal levels of infiltrating lymphocytes and the perivascular infiltrates are rare in the cerebral cortex. This can indicate that the demyelination process in the grey matter may not be exclusively immune-mediated. (34)

In MS, the astrocytes produced via astrogliosis situated in the white matter of the brain are deficient in β2 adrenergic receptors, and this lack of receptors makes the negative feedback control of pro-inflammatory cytokines carried out by norepinephrine

impossible, leading to an increment of the inflammatory state and subsequent inhibition of axon growth and remyelination in brain lesions (16) (5)

Astrogliosis is a complex response to CNS injuries and neurodegeneration that involves proliferation, morphological changes and functional adaptation of astrocytes. (4) Astrocytes are the most numerous glial cell type. They have a wide range of functions, especially in relation to brain homeostasis and neuroprotection, and are also the most important source of inducible Nitric Oxide Synthase (iNOS) in neuroinflammation (being a producer of nitric oxide (NO) in response to a wide variety of stimuli). The NO produced by astrocytes plays a very important role in MS, as it is the most prominent damage-inducing molecule in neuroinflammation.(35) By maintaining cell-to-cell interactions with neurons, astrocytes may also perform an important role in the regulation of brain inflammation. (36) (37) The involvement of astrocytes in modulating neuroinflammation also involves its ability to prevent microglial over activation and apoptosis. (38)

Besides astrocytes, microglia cells also play an important role in neuroinflammation. They are the resident macrophage-like cells of the CNS, with a broad role in the brain's innate and adaptive immunities and heterogeneous constitution as a cell line. Despite the similarities between microglia and various other tissue-resident macrophages, they can be distinctive from the rest by their restricted prenatal origin and their capacity for self-renewal and longevity. (39) Microglia show great functional plasticity when activated, and are equipped with a broad range of pattern-recognition receptors to detect microbial intruders and brain damage. (40) Depending on the stimuli, macrophages and microglia can undergo either classical activation (M1) or alternative activation (M2). M1 activation is a proinflammatory and neurotoxic state typically induced by triggering TLR's and IF-*γ* signalling pathways. This will lead to the production on proinflammatory cytokines such as TNFα, IL-1β or IL-12, and could also lead to a increase of NO production. On the other hand, M2 activation describes the anti-inflammatory and tissue remodelling activities of macrophages and microglia. (41) Microglia constitutes the first line of defence in the CNS, and their varied acute mediators can subsequently activate astrocytes. Nonetheless, microglia play not only a part in neuroinflammation but also in classical toxicity, where they work both as modulators and as indicators of damage. (39)

1.2.3 The role of Endothelin in the Context of MS

Endothelin-1 (Et-1) is a potent vasoconstrictor widely distributed in the body, including the CNS and specially the neurons and glia cells. (42) Et-1 is known to activate inflammatory transcription factors like Nuclear factor kappa-light-chain-enhancer (NF-κB) and up-regulate the expression of proinflammatory cytokines like TNFα and IL-1β (both present in high concentrations in MS sclerotic plaques). These transcription factors and cytokines in turn, stimulate Et-1 production. T cells are proven to stimulate the production of Et-1 by monocytes via secondary factors such as TNFα.(43) Previous studies defend that Et-1 is synthesized by both macrophages and dendritic cells as well as monocytes.(17) (44) The ET-1 produced by activated astrocytes can lead to long-lasting vasoconstriction, alas setting the stage for chronic hypoperfusion of the CNS. By resorting to animal models, it is possible to affirm that chronic hypoperfusion of the CNS induces mitochondrial energy failure and oxidative stress, leading to the release of oxygen reactive species and subsequent neuronal death.(44) (45)

Some epidemiological studies have reported vascular abnormalities and impairments in patients with MS (46), and defend that patients with MS have an increased risk of developing ischemic stroke and other vascular impairments. Cerebral hypoperfusion has been associated with MS for a long time, and has also been linked to chronic hypoxia, diffuse axonal degeneration, focal lesion formation and cognitive impairment. (10) It is generally believed that high levels of ET-1 in the cerebral circulation might be responsible for the Cerebral Blood Flow (CBF) changes that give origin to such impairments. Et-1 is usually produced by vascular endothelial cells and released extraluminally to regulate local vascular tone, however, a small amount of Et-1 is released intraluminally, contributing to the increase of Et-1 circulating levels. This effect can add up with the ET-1 expressed by other cell types in specific conditions such as inflammation and lead to excessive vasoconstriction.(47)

Since reduced CBF appears to be an intrinsic part of MS, and present since the beginning of the diagnose, the study of Et-1 and its antagonists seems to be an interesting subject to explore in the research for new therapies for MS.

1.3 The Autoimmune Encephalomyelitis (EAE) Model for MS

The Experimental Autoimmune Encephalomyelitis (EAE) model was first described over 85 years ago, and is still the most popular and used *in vivo* model for MS. (48) EAE is a T-helper cell-mediated autoimmune disease characterized by T-cell and

monocyte infiltration in the CNS associated with local inflammation. The autoimmune molecular targets identified and utilized through most cases have been myelin-producing oligodendrocytes, resulting in primary demyelination of axons, impaired axonal conduction and progressive hind-limb paralysis. EAE has been a strong tool for studying MS pathogenesis as well as potential therapeutic interventions. (49) In fact, most of what has been described so far, has been identified, tested or confirmed in EAE. It has become impossible to discuss MS without referring this model as one of its most faithful representations and study tools. However, there are still some differences between the two pathologies. The main difference between MS and EAE is that the latter requires an external immunization step to develop, while in humans, the origin of the disease is not artificially induced. In EAE, the sensitization of T cells to myelin antigens usually occurs through the use of an adjuvant (usually containing bacterial factors) highly capable of activating the innate immune system. This means that in EAE, the inducing antigens are known, whereas in MS there isn't an unique identified trigger. (16)

Like all animal models, EAE has limitations when applied to human MS disease. Thanks to its very heterogeneous induction methods, clinical and pathological features and amenability to treatment, it expresses an inherent complexity that may hinder its usefulness. This makes it essential to adjust this model to the specific scientific or clinical question that is being addressed. (50) (16)

1.4 Current Therapies and the role of Fluoxetine and Simvastatin

During the past decades, there have been conducted over 50 phase II and phase III clinical trials (including PMS patients), and they have achieved substantial progresses in finding disease modifying treatments (DMTs) for MS. Most of these treatments have proven themselves useful in the amelioration of the symptoms of RRMS patients, but PMS is still left without a viable treatment that significantly slows down the progress of the disease. (13) (14)

In Portugal, the most recurring therapies used in the treatment of MS cases are the following DMTs: interferon β (IFN β) formulations, Glatiramer Acetate (GA), Alemtuzumab and Fingolimod.(51) (52)

IFNs are natural antiviral molecules produced by a variety of cells, and their pharmacological interest relies on their immunoregulatory properties. Additionally, GA is a copolymer of four amino acids present in myelin structural proteins such as glutamic acid, lysine, tyrosine and alanine, and it affects the cytokine expression

pattern with its immunomodulatory effect. (5) Alemtuzumab has proven itself effective as a second line option, for MS patients with grave symptoms, as it is an Anti-CD52 monoclonal antibody, leading to a depletion of immune cells presenting CD52 surface marker (acting as an immunosuppressant, accomplishing a supressing effect on RRMS) (53) Fingolimod is a sphingosine 1-phosphate receptor modulator, and by blocking this receptor in T cells, inhibits their migration from the lymphoid tissue into the central nervous system and peripheral circulation. (54)

Despite being effective in reducing the relapse rate in RRMS up to roughly 30%, these have little to no impact on any PMS scenarios. (16) This said, it is of the utmost importance to continue investigating possible therapeutic agents and confirm any suspects of off-label uses of already known therapies.

Fluoxetine and Simvastatin are amongst the already known therapies thought to have a beneficial effect on MS, currently under investigation. (13)

Fluoxetine is a Selective Serotonin Reuptake Inhibitor (SSRI), commonly prescribed as an antidepressant. It has been highly suggested that Fluoxetine has neuroprotective properties by suppression of microglia activation and production of NF-κB by activated B cells, one of the most important transcription factors in inflammatory responses and cytokine production activity. (55) As a SSRI, Fluoxetine increases the concentration of serotonin (5-hydroxytryptophan, 5-HT) and helps to regulate the serotonergic system. It is thought that this increase of 5-HT also contributes to this drug's anti-inflammatory properties, as most immune system cells show at least one receptor for it (5-HTR), crucial to their activation and regulation mechanisms.(56) (25) (55)

Simvastatin is a statin (hydroxymethylglutaryl-CoA reductase inhibitors), and as such, it is vastly prescribed for hypercholesterolemia.(57) However, this pharmacological class is also hinted to possess immunomodulatory and neuroprotective properties and has been shown to improve cerebrovascular hemodynamics and modulate the expression of MHC. (58) These properties make statins an interesting candidate drug in patients in later stages of PMS, when dysfunction of brain parenchymal cells and vascular endothelial cells occur. Simvastatin is specially promising, because of its pharmacological potency and higher capacity of crossing the blood-brain barrier.(13) (59) Some EAE studies have shown that there is a decrease of disease activity and whole brain atrophy when comparing subjects treated with Simvastatin with those in

the placebo groups, supporting the study of this drug as a possible DMT for PMS. (14) (59)

In previous work (hasn't yet been published), Dr. Stephanie Hostenbach (VUB) has concluded that there exists an inverse ratio between the produced levels of Endothelin-1 and the concentration of Simvastatin and Fluoxetine in stimulated cells. In extension of this study, it was sought to further understand the inhibitory pathways in which Fluoxetine and Simvastatin carry out their influence in the expressed levels of Endothelin-1 in brain cell lines under physiological stress. Cellular stress was induced resorting to starvation, a well-known stress factor of the CNS cells. (61) This state was achieved by switching the growth culture medium to a Glutamine-free medium.

The Human Immortalized Astrocytoma cell line 1321N1, already documented for expressing high levels of Et-1 receptors (60) and the Mouse Immortalized murine neonatal Microglia cell line BV2, the most used and well characterized substitute for primary microglia in *in vitro* studies (40), are *in vitro* models of considered relevance in our days. Both cell lines are thought to be capable of producing Endothelin-1 under specific stimuli. (62) Considering the nature and objective of the experiment, it was decided to proceed with an *in vitro* model (instead of a classic EAE *in vivo* model) since it is more appropriate for the short time window available and because it spares mouse lives.

2 Objectives

Given the current scenery concerning Multiple Sclerosis and other neurodegenerative diseases, finding new ways of treatment presents itself as a mandatory task. In order to do so, it is essential for the scientific community to pursuit further understanding of its physiopathology and the mechanisms involved in the progression of the disease, as well as the specific pathways that are involved in today's therapies.

As previous results obtained by Dr. Stephanie Hostenbach (unpublished) refer, there is a visible negative correlation between the levels of produced Endothelin-1 in Microglia (Mouse BV2) as well as in Astrocytoma (Human 1321N1) cells *in vitro*, and the stimulation of the cell cultures with Fluoxetine and Simvastatin. Now, our focus is to understand the underlying mechanism that justifies this inhibition.

This experiment aims to understand the role of Simvastatin and Fluoxetine as neuroprotective drugs, by finding eventual correlations between their presence and the levels of pro inflammatory cytokines produced by CNS cells under stress, whom are thought to lead to a higher expression of Endothelin-1, a powerful vasoconstrictor, in cerebral tissue.

Considering the nature and objective of the experiment, it was decided to proceed with an *in vitro* model (instead of a classic EAE *in vivo* model) since it is more appropriate for the short time window available and also because it spares mouse lives. For this, the selected cell lines were the Mouse Immortalized murine neonatal Microglia cell line BV2, the most used and well characterized substitute for primary microglia in *in vitro* studies (40) and the Human Immortalized Astrocytoma cell line 1321N1, already documented for expressing high levels of Et-1 receptors (60).

3 Materials and Methods

3.1 Materials

3.1.1 Lab Equipment and Mediums

- Laminar Flow Cabinet - LabGard ES 425 (Nuaire)
- Eppendorf™ 5810R Centrifuge
- New Brunswick™ Innova 2000 Platform Shaker
- Nunc™ Cell-Culture Treated 12-Well Plate
- Nunc™ Cell-Culture Treated 24-Well Plate
- Nunc™ EasYFlask™ Cell Culture Flasks, T175, solid cap
- Nunc™ Cell Scrapers
- Cell Incubator (37ºC, 5%CO_2)
- 50 mL Sterile Falcon Tubes
- 4 mL Plastic Tubes with cap
- 1 mL Eppendorf Tubes
- 2 mL Eppendorf Tubes
- Sterile Glass Pipettes
- Pipettes and Pipette tips
- Microscope Olympus CK40
- Microplate Reader Bio Rad Model 680
- DMEM GlutaMAX™ with phenolred/L-Glutamine with 10% FBS (Fetal Bovine Serum), 1% PenStrep (Penincillin-Streptomycin) and 1% Fungizone (Amphotericin B)
- DMEM without phenolred/L-Glutamine with 10% FBS (Fetal Bovine Serum), 1% PenStrep (Penincillin-Streptomycin) and 1% Fungizone (Amphotericin B)
- RPMI 1640 Medium without phenolred/L-Glutamine with 10% FBS (Fetal Bovine Serum), 1% PenStrep (Penincillin-Streptomycin) and 1% Fungizone
- PBS 1% solution (Sigma-Aldrich)
- Trypsin 1% (10x diluted in PBS) (Sigma-Aldrich)
- Invitrogen Human IL-1 beta Uncoated ELISA kit (Thermo Fisher)
- Invitrogen Human TNF alpha Uncoated ELISA kit (Thermo Fisher)
- Invitrogen Mouse IL-1 beta Uncoated ELISA kit (Thermo Fisher)
- Invitrogen Mouse TNF alpha Uncoated ELISA kit (Thermo Fisher)

3.1.2 Cell lines

- Human Astrocytoma 1321N1 cell line.
- Mouse Microglia BV2 cell line. For this experiment, two different groups of cells were used: BV2 p6 (originally obtained from a cell culture undergoing its sixth passage) and BV2 p18 (originally obtained from the same cell culture, but undergoing its eighteenth passage).

3.1.3 Drugs

- Simvastatin (Merck) solution of 1µM, 5 µM and 25 µM concentration (stock solution 50mM). DMSO was used for the preparation of all Simvastatin solutions as a vehicle.
- Fluoxetine (Lilly) solution of 1 µM, 5 µM and 10 µM concentration (stock solution 10mM) Milli-Q water was used for the preparation of all Fluoxetine solutions as a vehicle.

3.2 Methods

During this study there were made several protocol changes, as means to improve the reliability of the obtained results.

3.2.1 Cell manipulation

Each cell line was suspended at a density of $3x10^4$ cells/mL in a DMEM with phenolred/L-Glutamine (suplemented with 10% FBS, 1% PenStrep and 1% Fungizone), and cultured in a cell flask in a humidified 5% CO_2 atmosphere at 37ºC.

3.2.2 Counting of cells

Before each Cell line passage, the cells of each individual flask were counted in a Bürker Chamber, using 10µl of the homogenized cell culture medium and 90µl of trypane blue solution.

3.2.3 Cell culture and stimulation

Cells were cultured in 12 well plates in a humidified 5% CO_2 atmosphere at 37ºC. Each well contained 30.000 cells suspended in 2mL of growth medium (DMEM with phenolred/L-Glutamine, 10% FBS, 1% PenStrep and 1% Fungizone). After 72 hours, the plate was centrifuged for 5 minutes at 15000 rpm, and the culture medium exchanged for a starvation medium (DMEM without phenolred/L-Glutamine but with 10% FBS, 1% PenStrep and 1% Fungizone). The plate was then returned to the incubator for 24 hours. Next, the supernatant was removed using a vacuum system and replaced with 2mL of the stimulus solution (constituted of starvation medium and the study compound solution).

3.2.4 Cell harvesting

After 24 hours of stimulus the plate was centrifuged for 10 minutes at 14000 rpm, and the supernatant was transferred into an Eppendorf tube. The remaining cells were scrapped with a cell scrapper into 1mL of a cold PBS solution. The resulting solution was after transferred to an Eppendorf tube.

All resulting samples were frozen and stored at -80ºC until use.

3.2.5 Protocol alteration

Cells were cultured in 24 well plates in a humidified 5% CO2 atmosphere at 37ºC. Each well contained 1.000.000 cells suspended in 1mL starvation medium (DMEM without phenolred/L-Glutamine but with 10% FBS, 1% PenStrep and 1% Fungizone). After 24 hours the supernatant was removed using a vacuum system and replaced

with 1mL of the stimulus solution (constituted of starvation medium and the study compound solution). Cell harvesting was performed after 6 or 24 hours, using the same procedure as used before.

3.2.6 Cytokine assays

Cytokine levels were quantified by enzyme-linked immunosorbent assays (ELISAs) using cytokine and cell type specific uncoated ELISA kits from Invitrogen. ELISAs were carried out according to the manufacturer's protocol using the following antibody concentrations: TNFα coating antibodies total concentration 1.0 µg/mL; TNFα detection antibody 0.25 µg/mL; IL-1β coating antibodies total concentration 1.0 µg/mL; IL-1β detection antibody 0.5 µg/mL. The sensitivities of the ELISA's for TNFα and IL_1 β were 10 pg/mL.

- The ELISA plates were read on the Microplate Reader on the Dual Reading mode, using the 450nm wavelength as the Measurement Filter and the 540nm wavelength as the Reference Filter.

3.2.7 Statistical analysis

Statistical comparisons were made using a one-way ANOVA with a Tukey post-test. A probability value less than 0.01 (p<0,01) was considered significant. Statistical analysis was made using GraphPad Prism™ version 6.04.

Results were obtained from at least 4 independent experiments and are expressed as mean±SEM.

4 Results

To evaluate the effect of Simvastatin (Simv) and Fluoxetine (Fluox) on the production of pro-inflammatory cytokines IL-1β and TNFα (by ELISAs), cells were exposed to several drug concentrations at different cell culture conditions ($3x10^4$/ml and $1x10^5$/ml cells plated throughout the course of 13 passages). These essays were applied to every collected medium sample (obtained after a 6- or 24-hour cell stimulation). To assess the viability of the results, samples collected from the main stimulation solutions were also tested (at timepoint 0 hours). In the following graphics, the data will be shown in the following order: Medium, Vehicle, Fluoxetine/Simvastatin (and respective concentrations), and a second set of results for each group (0h). The Medium column displays the results obtained from the cell cultures where it was added the starvation medium (without any additional substance). The Vehicle column displays the results obtained from the cell cultures where it was added starvation medium with the vehicle used to dilute the stimulus drug (DMSO for Simvastatin and Milli-Q water for Fluoxetine). The “Simv” and “Fluox” columns display the results obtained from the cell cultures where it was added the Simvastatin and Fluoxetine stimulus solutions respectively, each for a respective concentration of drug. The next columns correspond to the results obtained from analysing all the initial solutions (before adding them to the cell culture) used throughout the cell stimulation process: the medium, vehicle and stimulation solutions. These are identified by the “(0h)” in their column name, because they reflect the integrity of the solution before added to the culture medium.

In order to understand the origin of the recurrent interferences shown on most of the essays, another ELISA was also carried through for the principal compounds of the culture and stimulation mediums.

4.1 Human Astrocytoma 1321N1 cell line

To access the effect of Fluoxetine and Simvastatin in the 1321N1 cell line, cell cultures were stimulated with different concentrations of these drugs and lysed. The supernatant obtained from the lysis procedure was then collected and analysed in ELISAs as previously described. Plating the 1321N1 cells in 12-well-plates didn’t show any correlation between the concentration of the drugs and the production of neither of the targeted cytokines and the signals obtained were very faint. (fig.1A) As

a way to obtain stronger signals, cell cultures were plated in 24-well-plates with a higher cell count (fig. 1B). Still, Simvastatin and Fluoxetine didn't produce relevant data for neither IL-1β (fig. 1B and fig. 1C) or TNFα (fig. 1D).

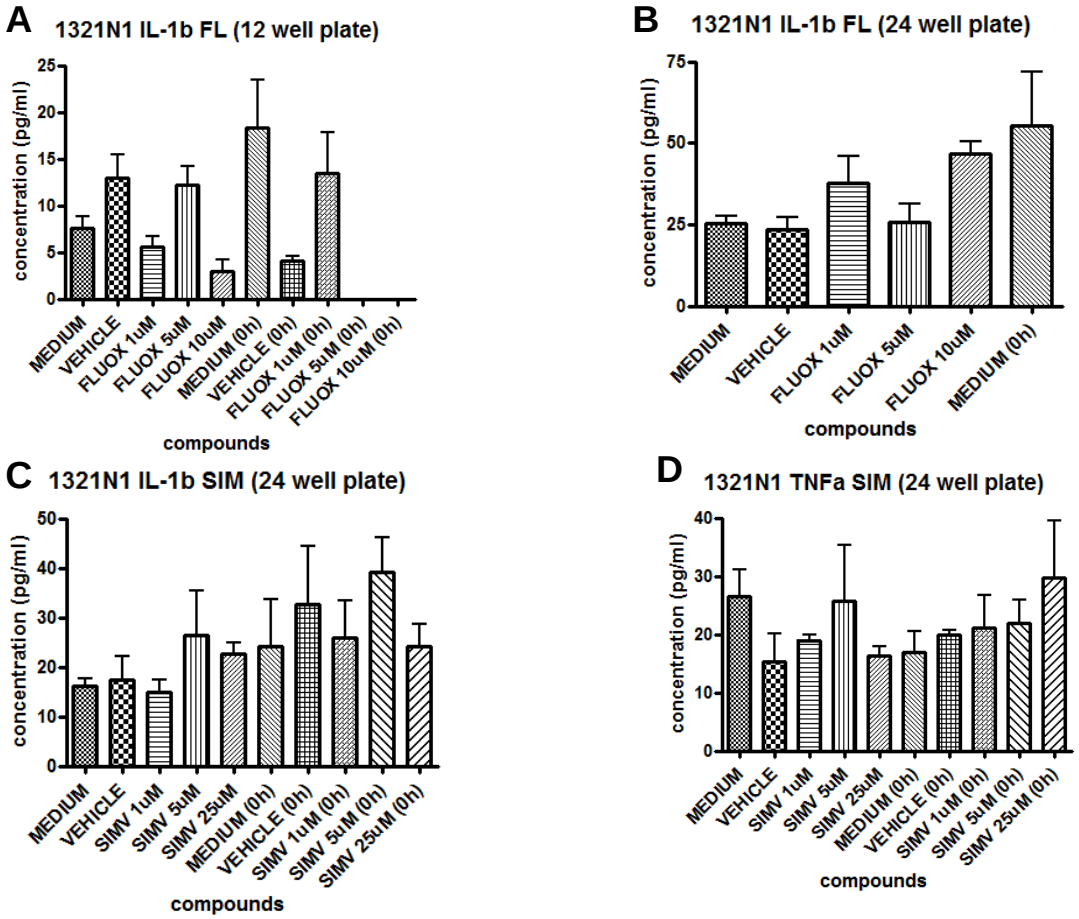

Fig. 1. (A) The effect of Fluoxetine on the expression of IL-1β in 1321N1 human cells cultured on a 12 well plate (with approximately 30 000 cells per well). 1321N1 cells were cultured under the same conditions and stimulated with 3 different concentrations of Fluoxetine (1µg, 5µg and 10µg). The histogram depicts the concentration of IL-1β produced for each concentration of Fluoxetine and compares it with negative controls; (B) The effect of Fluoxetine on the expression of IL-1β in 1321N1 human cells cultured on a 24 well plate (with approximately 1 000 000 cells per well). 1321N1 cells were cultured under the same conditions and stimulated with 3 different concentrations of Fluoxetine (1µg, 5µg and 10µg) The histogram depicts the concentration of IL-1β produced for each concentration of Fluoxetine and compares it with negative controls; (C) The effect of Simvastatin on the expression of IL-1β in 1321N1 human cells cultured on a 24 well plate (with approximately 1 000 000 cells per well). 1321N1 cells were cultured under the same conditions and stimulated with 3 different concentrations of Simvastatin (1µg, 5µg and 25µg) The histogram depicts the concentration of IL-1β

produced for each concentration of Simvastatin and compares it with negative controls; (D) The effect of Simvastatin on the expression of TNFα in 1321N1 human cells cultured on a 24 well plate (with approximately 1 000 000 cells per well). 1321N1 cells were cultured under the same conditions and stimulated with 3 different concentrations of Simvastatin (1µg, 5µg and 25µg) The histogram depicts the concentration of IL-1β produced for each concentration of Simv and compares it with negative controls.

The negative controls used for every ELISA consisted of MEDIUM (cells + starvation medium), VEHICLE (cells + starvation medium + vehicle in which the study compound was originally dissolved) and the "0h" samples of every solution added to the cell culture. The results obtained are affected by unusually high signals on the negative controls. In addition, Throughout the experiment, there were no conclusive results obtained from the 1321N1 cell type. The statistical analysis showed that the presence of Simvastatin and Fluoxetine didn't cause any measurable increase or decrease on the levels of produced IL-1β or TNFα.

4.2 Mouse Microglia BV2 cell line

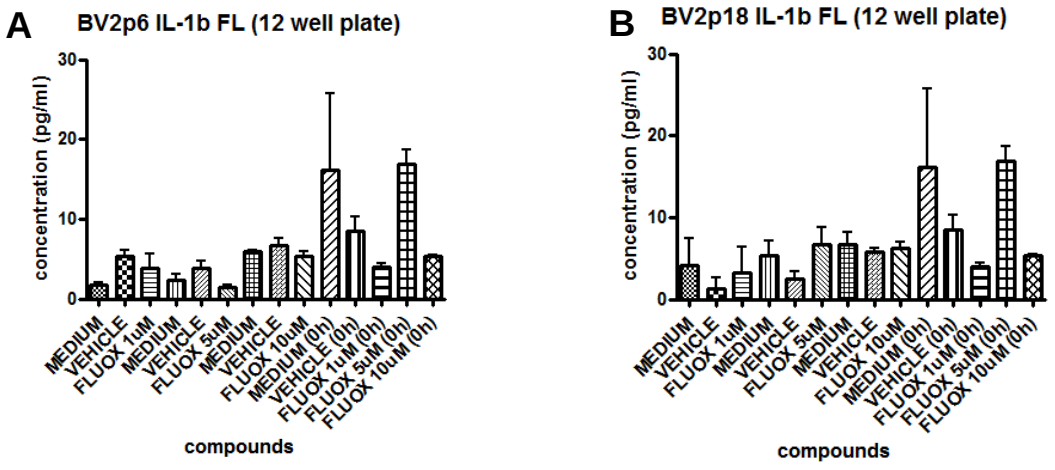

Fig. 2. (A) & (B) The effect of Fluoxetine on the expression of IL-1β in BV2 mouse cells cultured on a 12 well plate (with approximately 30 000 cells per well). BV2 cells were cultured under the same conditions and stimulated with 3 different concentrations of Fluoxetine (1µg, 5µg and 10µg). These histograms depict the concentration of IL-1β produced for each concentration of Fluox and compare it with negative controls.

In order to study the immune response of BV2 cell lines when stimulated with Fluoxetine or Simvastatin, there were used two different populations of BV2 cells. One of the cell lines was obtained from a pre-existing cell culture currently undergoing its 6th passage (BV2p6) while the other was obtained from an also pre-existing cell culture currently undergoing its 18th passage (BV2p18). The results obtained are both presented in Fig. 2A and 2B, respectively.

To evaluate the inhibitory effect of Fluoxetine in the production of IL-1β in BV2 mouse cells, the cell cultures were plated in 12 well plates and later stimulated with solutions of different concentrations of this drug and lysed after a 24-hour period. The supernatant obtained from the lysis procedure was then collected and analysed in ELISAs as previously described. The initial results obtained for IL-1β were inconclusive when stimulating the cells with Fluoxetine, as the negative controls indicated high interferences in the solutions and the signals were week for both cell cultures. (fig. 2A and fig. 2B)

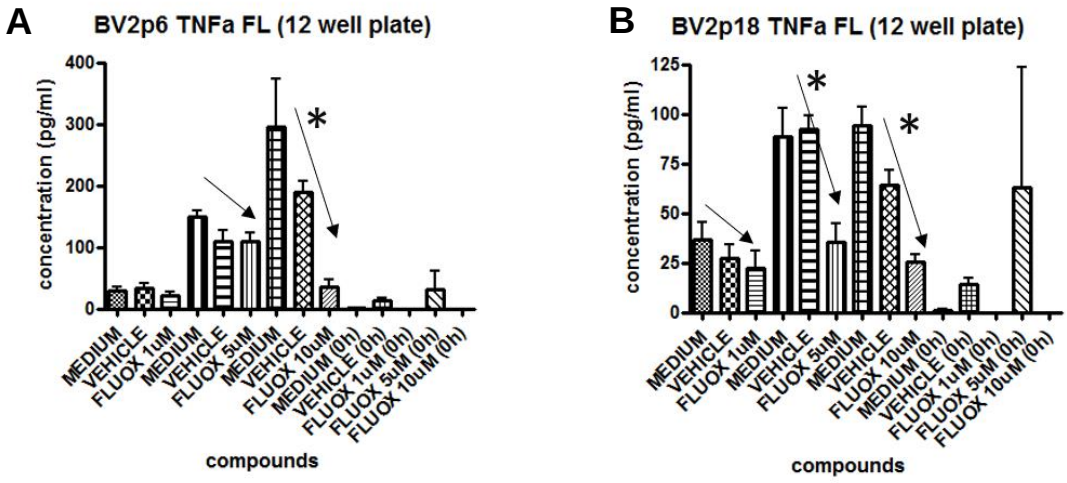

Fig. 3. (A) & (B) The effect of Fluoxetine on the expression of TNFα in BV2 mouse cells cultured on a 12 well plate (with approximately 30 000 cells per well). BV2 cells were cultured under the same conditions and stimulated with 3 different concentrations of Fluoxetine (1µg, 5µg and 10µg). These histograms depict the concentration of TNFα produced for each concentration of Fluox and compare it with negative controls. Significance is indicated by * (p <0,01).

The results obtained after the stimulation of mouse cells with Fluoxetine show that there is a significant decrease of the signal and therefore of TNFα production on the

samples stimulated with this compound. The signal inhibition is higher for higher concentrations of Fluoxetine, and it is possible to verify the significant (p<0,01) results, especially for higher concentrations of the stimulus solution (fig. 3A and fig. 3B). The presence of interferences in the negative controls was significantly lower than what was observed in previous essays.

After obtaining the first set of results recurring to the 12 well plating technique, the BV2 cell lines were plated in 24 well plates, with a higher cell count. The cells were submitted to the previously mentioned protocol and stimulated with different concentration solutions of Simvastatin. The samples obtained from said cultures were then analysed in ELISAs (fig. 4).

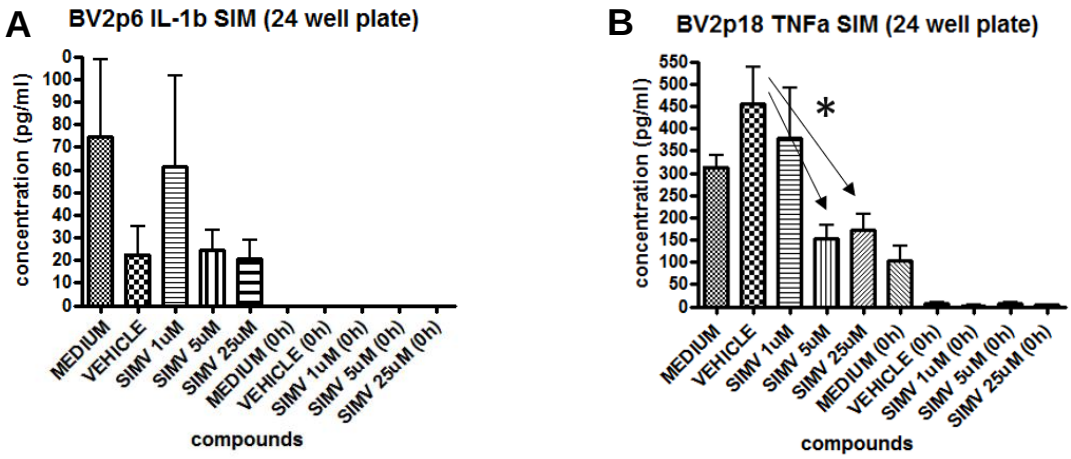

Fig. 4. The effect of Simvastatin on the expression of TNFα and IL-1β in BV2 mouse cells cultured on a 24 well plate in DMEM and RPMI Medium (A) & (B) with approximately 1 000 000 cells per well. BV2 cells were cultured under the same conditions and stimulated with 3 different concentrations of Simvastatin (1µg, 5µg and 25µg). These histograms depict the concentration of IL-1β and TNFα produced for each concentration of Simv and compare it with negative controls. Significance is indicated by * (p <0,01).

Although the results obtained seemed to suggest a correlation between the concentration of IL-1β and the concentration of the Simvastatin solutions used (fig. 4A), further data analysis showed there was no significance between the values

obtained (p>0,01). The results obtained after the Simvastatin stimulation of BV2 cells using RPMI medium, show that there is a significant decrease of the signal (therefore of TNFα production) on the samples stimulated with this compound (fig. 4B). The signal inhibition is higher for higher concentrations of Simvastatin, and it is possible to verify the significant (p <0,01) results, especially for higher concentrations of the stimulus solution.

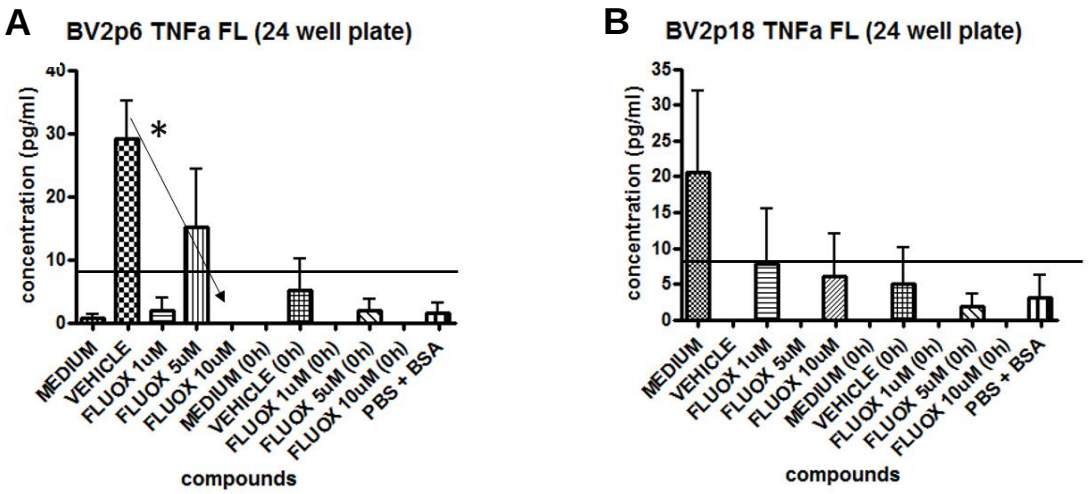

Fig. 5. The effect of Fluoxetine on the expression of TNFα in BV2 mouse cells cultured on a 24 well plate in DMEM and later PBS as the starvation medium (A) & (B). BV2 cells were cultured under the same conditions and stimulated with 3 different concentrations of Fluoxetine (1µg, 5µg and 10µg) for 6 hours. These histograms depict the concentration of TNFα produced for each concentration of Fluox and compare it with negative controls. Significance is indicated by * (p <0,01).

To assess the inhibitory role of Fluoxetine in the production of TNFα in BV2 cell cultures in the 24 well plates, an additional experiment was carried through. Due to the lack of time, cell cultures were put through a 6-hour short stimulus in DMEM medium (fig. 5A) and PBS (fig. 5B) using the usual Fluoxetine solutions.

Although detectable, most of the signals obtained from the 6-hour stimulation are lower than the lowest point on the Standard Curve made for each ELISA plate, meaning the results are susceptible to a degree of uncertainty. The results obtained after the Fluoxetine stimulation of BV2 cells using a 6-hour stimulation period, and

PBS as medium, show that there is a significant decrease of the signal (therefore of TNFα production) on the samples stimulated with this compound (fig. 5B). However, the data obtained from the 24-hour stimulation with Fluoxetine on the 24 well plates wasn't viable, as it showed signs of great interferences on the negative controls.

After obtaining the already shown results on the cell culture samples, it became clear that there was a need to investigate the origin of the observed interferences transversally present in all the ELISAs.

Consequentially, an ELISA was conducted on each individual compound and solution as a mean to assess the origin of the recurrent interferences observed throughout the experiment (fig 6). The compounds were analysed in their initial conditions, without having any contact with cell cultures or further manipulation. Most of the results obtained had a signal intensity below the limit of detection.

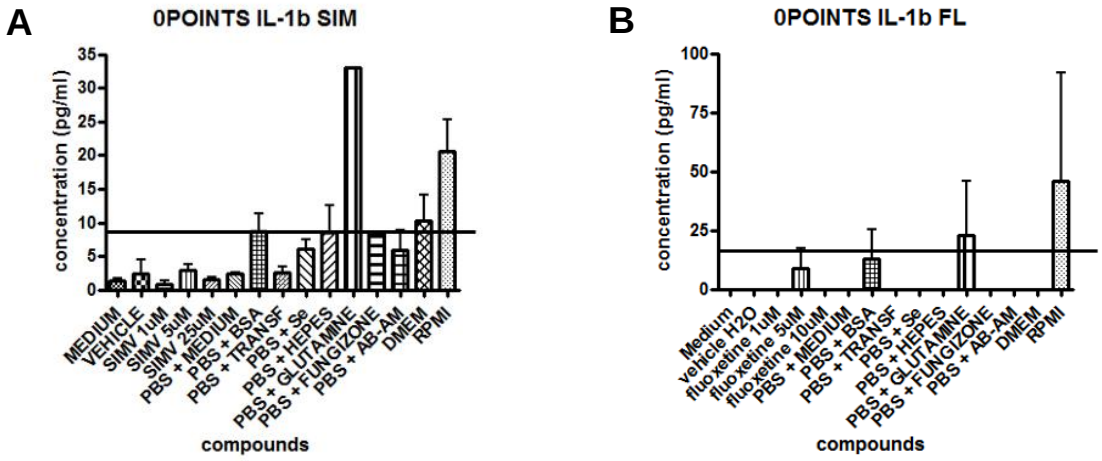

Fig. 6. Data obtained from analysing every compound or solution used during the cell stimulation protocol. The horizontal line represents the Lowest point on the Standard Curve made for each ELISA.

Although there weren't conclusive results for the TNFα signal interferences, the histograms suggest IL-1β signals seem to be affected by the presence of RPMI medium and Glutamine.

5 Discussion

Fluoxetine and Simvastatin are amongst the already known therapies thought to have a beneficial effect on MS and are currently under investigation, so that their biological pathways can be studied. (13) In this study, the stimulation of cells with Fluoxetine and Simvastatin (and following measurements of produced levels of pro-inflammatory cytokines TNFα and IL-1β by microglia and glial cells) was used as a strategy to further understand the role of these drugs in neuroinflammation. The outcomes from this study may help understanding the right path to take, in terms of finding alternative and innovative solutions and treatments for MS.

Although there were no conclusive results related to the Human 1321N1 astrocytoma cell line, the obtained results suggest that the experiments should be carried out in different conditions with the objective to minimize interferences and optimize cell growth and harvesting, since it is believed that astrocytes play an important role in cellular signalling and modulating inflammation (therefore, should show responses to the stimulation with cytokine production inhibitors).(35) The interferences seen when assessing the cytokines on the blanks (both medium used in 1321N1 cells and vehicle) may also being originated from the ELISA protocol followed. Additionally, the absence of relevant signals may indicate that cellular integrity wasn't assured in the cell culture.

The results obtained with the mouse BV2 microglia cell line suggested that Fluoxetine could induce an inhibitory effect on the production of TNFα, supporting the results widely obtained in other experiments. (6) (55) These results also suggested that Simvastatin could induce an inhibitory effect on the production of both TNFα and IL-1β, although there is a lack of significant data to support the latter case. The role of Simvastatin is still controversial, as there are reports defending that statins are ineffective in cases of neuroinflammation, (57) while others affirm that neuroinflammation and CNS function improve after treatment with Simvastatin. (59) These results also prove that the BV2 cell line is indeed a trustworthy tool as an alternative model for primary microglia cultures in the study of brain inflammation (40)

Regarding the methods applied on this experiment, the plating of a higher number of cells (approximately 1 000 000 cells per well) helps obtaining stronger signals, and therefore, more trustable results. The cell culture methods utilized should be adapted to the cell culture state. During this experiment, the 1321N1 cell line started to show less adherence to the culture flask. Protocols were carried out as usual, leading to a

significant loss of cells during the medium exchange, leading to weaker signals and data bias. For future reference, there should be made procedural adjustments to minimize possible error sources.

The source of interferences remained unidentified, as the assays done to the compounds didn't show a specific source for the high signals obtained on the negative controls. This uncertainty can severely harm the experiment's credibility and should be addressed in the future.

Dr. Stephanie Hostenbach's results showed in the past how the levels of endothelin increase in the presence of TNFα and IL-1β and decrease when the studied cell lines are stimulated with Simvastatin and Fluoxetine. The results obtained in the present experiment with mice cell line indicates that this decrease could be due to an inhibition of the production of these pro inflammatory cytokines by the analysed drugs, and not by other competitive mechanisms. One step further for validating the now obtained results when mice cell line are exposed to fluoxetine or simvastatin is assessing the human cell lines response to those drugs. The fact that there were results in the mouse cell line, but not in the human cell line affect the overall relevance of this study, and makes it crucial that the experiments with human cells are repeated, since there is no tangible conclusion explaining the lack of results or presence of interferences. Human cell lines are harder to replicate than mouse cells, and their maintenance is harder to perform. (16) This may be one of the reasons why there were no results in the 1321N1 cell line, as the cells integrity may have been compromised along the experimental procedure (as suspected). As an alternative, another human cell line could be used as an *in vitro* model for the monitorization of the anti-inflammatory effect of Simvastatin and Fluoxetine.

6 Conclusion

With the increasing number of diagnosed cases of neurodegenerative diseases, it is of the utmost importance to understand the biological pathways that trigger these kinds of pathologies. To do so, it is crucial to study the causes and effects of neuroinflammation as well as possible means to treat and prevent it. This study's objective was to understand the effect of Fluoxetine and Simvastatin on the production of IL-1β and TNFα in cells under stress, as these proinflammatory cytokines are thought to increase the expression of Endothelin-1. By stimulating human and mouse brain cell lines with Fluoxetine and Simvastatin and analysing the effect on cytokine (IL-1β and TNFα) production levels, it was intended to assess if these drugs have any specific effect on the production of those proinflammatory cytokines, and if so, a possible link between these drugs, Fluoxetine and Simvastatin, and the expression of Endothelin-1 in CNS cells under stress could be envisioned.

The obtained preliminary results suggest that Fluoxetine and Simvastatin seem to have an inhibitory effect of the proinflammatory cytokines IL-1β and TNFα on the mouse BV2 cell line. However, the results obtained are not free from considerable interferences, and so further work is required as well as process optimization procedures. Until these uncertainties are resolved, there is a chance that Fluoxetine and Simvastatin could have an inhibitory effect on the production of Endothelin-1 on the mouse BV2 cell line through modulation of these proinflammatory cytokine levels. Without confirmation of these findings on human cells the biological pathway responsible for this inhibitor effect, and its therapeutic usability, remains unfound.

7 References

1. World Health Organization. World Health Report @ www.who.int [Internet]. 2001 [cited 2019 Sep 5]. Available from: https://www.who.int/whr/2001/media_centre/press_release/en/
2. Alzheimer's Statistics 2019 @ www.alzheimers.net [Internet]. [cited 2019 Sep 5]. Available from: https://www.alzheimers.net/resources/alzheimers-statistics/
3. Prevalence and incidence of multiple sclerosis @ www.mstrust.org.uk [Internet]. Last Updated November 2018. [cited 2019 Sep 5]. Available from: https://www.mstrust.org.uk/a-z/prevalence-and-incidence-multiple-sclerosis
4. Hostenbach S, Cambron M, D'haeseleer M, Kooijman R, De Keyser J. Astrocyte loss and astrogliosis in neuroinflammatory disorders. Neurosci Lett. 2014;565:39–41. Available from: http://dx.doi.org/10.1016/j.neulet.2013.10.012
5. Hauser SL, Oksenberg JR. The Neurobiology of Multiple Sclerosis: Genes, Inflammation, and Neurodegeneration. Neuron. 2006;52(1):61–76.
6. Lu Y, Ho CS, Liu X, Chua AN, Wang W, McIntyre RS, et al. Chronic administration of fluoxetine and pro-inflammatory cytokine change in a rat model of depression. PLoS One. 2017;12(10):1–14.
7. Rivera VM. Multiple Sclerosis: A Global Concern with multiple challenges in an Era of advanced therapeutic complex molecules and biological medicines. Biomedicines. 2018;6(4):1–8.
8. O'Gorman C, Lucas R, Taylor B. Environmental risk factors for multiple sclerosis: A review with a focus on molecular mechanisms. Int J Mol Sci. 2012;13(9):117:18–52.
9. Koch-Henriksen N, Sorensen P. Why does the north-south gradient of incidence of multiple sclerosis seem to have disappeared on the northern hemisphere? J Neurol Sci. 2011;311(1):58–63.
10. Rocha NP, Colpo GD, Bravo-Alegria J, Lincoln JA, Wolinsky JS, Lindsey JW, et al. Exploring the relationship between Endothelin-1 and peripheral inflammation in multiple sclerosis. J Neuroimmunol. 2019;326(8):45–8. Available from: https://doi.org/10.1016/j.jneuroim.2018.11.007
11. Ransohoff RM, Hafler DA LC. Multiple sclerosis-a quiet revolution. Nat Rev Neurol. 2015;11:134–42.

12. Lublin, F.D., Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. Neurology. 1996;46:907–911.

13. Shirani A, Okuda DT, Stüve O. Therapeutic Advances and Future Prospects in Progressive Forms of Multiple Sclerosis. Neurotherapeutics. 2016;13(1):58–69.

14. D'Amico E, Patti F, Zanghì A, Zappia M. A personalized approach in progressive multiple sclerosis: The current status of disease modifying therapies (DMTs) and future perspectives. Int J Mol Sci. 2016;17(10):1–14.

15. Bramlett HM, Dietrich WD. Pathophysiology of cerebral ischemia and brain trauma: Similarities and differences. J Cereb Blood Flow Metab. 2004;24(2):133–150.

16. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br J Pharmacol. 2011;164(4):1079–1106.

17. Shinagawa S, Okazaki T, Ikeda M, Yudoh K, Kisanuki YY, Yanagisawa M, et al. T cells upon activation promote Endothelin-1 production in monocytes via IFN-γ and TNF-α. Sci Rep. 2017;7(1):1–8. Available from: http://dx.doi.org/10.1038/s41598-017-14202-5

18. Ozenci V, Kouwenhoven M, Link H. Cytokines in multiple sclerosis: methodological aspects and pathogenic implications. Mult Scler. 2002;5:932–936.

19. Bielekova B, Martin R. Development of biomarkers in multiple sclerosis. Brain. 2004;127(7):1463–1478.

20. Okuda Y, Sakoda S, Saeki Y, Kishimoto T, Yanagihara T. Enhancement of Th2 response in IL-6-deficient mice immunized with myelin oligodendrocyte glycoprotein. J Neuroimmunol. 2000;105:120–123.

21. Cavanagh M, London SM. T-cell activation [Internet]. 2018 [cited 2019 Sep 13]. Available from: https://www.immunology.org/public-information/bitesized-immunology/systems-and-processes/t-cell-activation

22. Kasper LH, Shoemaker J. Multiple sclerosis immunology: The healthy immune system vs the MS immune system. Neurology. 2010;81:2–8.

23. Kutzelnigg A, Lucchinetti CF, Stadelmann C, Bruck W, Rauschka H, Bergmann M, et al. Cortical demyelination and diffuse white matter injury in multiple

sclerosis. Brain. 2005;128:2705–2712.

24. Jack C, Ruffini F, Bar-Or A, Antel JP. Microglia and multiple sclerosis. j Neurosci Res. 2005;81:363–373.

25. Bhat R, Mahapatra S, Axtell RC, Steinman L. Amelioration of ongoing experimental autoimmune encephalomyelitis with fluoxetine. J Neuroimmunol. 2017;313(8):77–81. Available from: https://doi.org/10.1016/j.jneuroim.2017.10.012

26. Friese MA, Fugger L. Pathogenic CD8(+) T cells in multiple sclerosis. Ann Neurol. 2009;66:132–141.

27. Trapp B., Nave K. Multiple sclerosis: an immune or neurodegenerative disorder? Annu Rev Neurosci. 2008;31:247–269.

28. Weber MS, Hemmer B, Cepok S. he role of antibodies in multiple sclerosis. Biochim Biophys Acta. 2011;239–245.

29. Bitsch A, Schuchardt J, Bunkowski S, Kuhlmann T, Bruck W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain. 2000;123:1174–1183.

30. Neumann H. Molecular mechanisms of axonal damage in inflammatory central nervous system diseases. Curr Opin Neurol. 2003;16:267–273.

31. Lassmann H, Bruck W, Lucchinetti CF. The immunopathology of multiple sclerosis: an overview. Brain Pathol. 2007;17:210–218.

32. Kutzelnigg A, Lucchinetti C., Stadelmann C, Bruck W, Rauschka H, Bergmann M, et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain. 2005;128:2705–2712.

33. Calabrese M, Filippi M, Gallo P. Cortical lesions in multiple sclerosis. Nat Rev Neurol. 2010;6(8):438–444.

34. Peterson JW, Bo L, Mork S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. Ann Neurol. 2001;50:389–400.

35. Mohsenzadegan M, Fayazi MR, Abdolmaleki M, Bakhshayesh M, Seif F, Mousavizadeh K. Direct immunomodulatory influence of IFN-β on human astrocytoma cells. Immunopharmacol Immunotoxicol. 2015;37(2):214–9. Available from: http://dx.doi.org/10.3109/08923973.2015.1014559

36. Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, et al. Astrocyte scar formation aids CNS axon regeneration. Nature. 2016;532(7598):195–200.

37. Brannvall K, Bergman K, Wallenquist U, Svahn S, Bowden T, Bra K. Multiple Mechanisms That Prevent Excessive Brain Inflammation. J Neurosci Res. 2007;2146:2138–2146.

38. Decker Y, McBean G, Godson C. Lipoxin A4 inhibits IL-1β-induced IL-8 and ICAM-1 expression in 1321N1 human astrocytoma cells. Am J Physiol - Cell Physiol. 2009;296(6):1420–1427.

39. Yin J, Valin KL, Dixon ML, Leavenworth JW. The Role of Microglia and Macrophages in CNS Homeostasis, Autoimmunity, and Cancer. J Immunol Res. 2017;1155(10):1–11.

40. Henn A, Lund S, Hedtjärn M, Schrattenholz A, Pörzgen P, Leist M. The Suitability of BV2 Cells as Alternative Model System for Primary Microglia Cultures or for Animal Experiments Examining Brain Inflammation Doerenkamp-Zbinden Chair for alternative in vitro methods to replace animal experiments. ALTEX. 2009;26(1):83–94.

41. Murray PG. Macrophage polarization. Annu Rev Physiol. 2017;79(1):541–566.

42. Cardell LO, Uddman R, Edvinsson L. Endothelins: A role in cerebrovascular disease? Cephalalgia. 1994;14(4):259–265.

43. Chang CW, Wu HC, Lyu RK, Lo YS, Chen CM, Ro LS, et al. Elevated serum levels of Endothelin-1 in patients with chronic inflammatory demyelinating polyneuropathy. Clin Chim Acta. 2018;476(5):49–53. Available from: https://doi.org/10.1016/j.cca.2017.11.008

44. Hostenbach S, D'haeseleer M, Kooijman R, De Keyser J. The pathophysiological role of astrocytic Endothelin-1. Prog Neurobiol. 2016;144:88–102.

45. D'Haeseleer M, Beelen R, Fierens Y, Cambron M, Vanbinst AM, Verborgh C, et al. Cerebral hypoperfusion in multiple sclerosis is reversible and mediated by Endothelin-1. Proc Natl Acad Sci U S A. 2013;110(14):5654–5658.

46. D'haeseleer M, Cambron M, Vanopdenbosch L, De Keyser J. Vascular aspects of multiple sclerosis. Lancet Neurol. 2011;10(7):657–666.

47. Flammer J, Konieczka K. The discovery of the Flammer syndrome: A historical and personal perspective. EPMA J. 2017;8(2):75–97.

48. Rivers T, Sprunt D, Berry G. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. J Exp Med. 1933;58:39–53.

49. Robinson AP, Harp CT, Noronha A, Miller SD. The EAE model of MS: utility for understanding disease pathophysiology and treatment. Hand Clin Neurol. 2014;122:173–189.

50. Miller S, McMahon E, Schreiner B, Bailey S. Antigen presentation in the CNS by myeloid dendritic cells drives progression of relapsing experimental autoimmune encephalomyelitis. Ann N Y Acad Sci. 2007;1103:179–191.

51. Furtado C. Utilização de novos medicamentos em Portugal @infarmed.pt. 2015;18–19.

52. Comissão Nacional de Farmácia e Terapêutica. Utilização de fármacos para o tratamento da esclerose múltipla. 2017;4:1–3.

53. Hu Y, Turner M, Shields J, Gale M, Hutto E, Roberts B, et al. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. Immunology. 2009;128:260–270.

54. Nicholas R, Giannetti P, Alsanousi A, Friede T, Muraro PA. evelopment of oral immunomodulatory agents in the management of multiple sclerosis. Drug Des Devel Ther. 2011;5:255–274.

55. Zhang F, Zhou H, Wilson B, Shi J, Hong J, Gao H. Fluoxetine protects neurons against microglial activationmediated neurotoxicity. Park Relat Disord. 2012;18(1):213–7.

56. Xia M, Li X, Yang L, Ren J, Sun G, Qi S, et al. The ameliorative effect of fluoxetine on neuroinflammation induced by sleep deprivation. J Neurochem. 2018;146(1):63–75.

57. Carroll JA, Race B, Phillips K, Striebel JF, Chesebro B. Statins are ineffective at reducing neuroinflammation or prolonging survival in scrapie-infected mice. J Gen Virol. 2017;98(8):2190–2199.

58. van der Most P, Dolga A, Nijholt I, Luiten P, Eisel U. Statins: mechanisms of neuroprotection. Prog Neurobiol. 2009;88:64–75.

59. Stein A, Stroobants S, Gieselmann V, D'Hooge R, Matzner U. Anti-

inflammatory therapy with simvastatin improves neuroinflammation and CNS function in a mouse model of metachromatic leukodystrophy. Mol Ther. 2015;23(7):1160–1168.

60. Griessmeier KJ, Müller CE. [3H]BQ-123 Binding to Native Endothelin ETA Receptors in Human Astrocytoma 1321N1 Cells and Screening of Potential Ligands. Pharmacology. 2005;74:51–56.

61. Philipp Mergenthaler, Lindauer Ute Gerald A. Dienel. Sugar for the brain: the role of glucose in physiological and pathological brain function. Trends in Neuroscience. 2013;36(10):587–597.

62. Anthony P, Davenport, Kelly A. et al. Endothelin. Pharmacological Reviews. 2016;68: 357-418.

8 Anexes

Declaração de Compromisso de Anti-Plágio

Eu, João Nuno Paiva Malvas, estudante n.º 9897 declaro por minha honra que o trabalho da minha autoria, intitulado *In Vitro Research on the role of vasoconstrictor Endothelin-1 in Neuroinflammation*, é original e que todas as minhas citações estão corretamente identificadas; no caso de ter utilizado frases de trabalhos de outros autores, ou, se as redigi com palavras diferentes, as fontes destas foram referenciadas devidamente. Tenho consciência de que a utilização de elementos alheios não identificados constitui uma grave falta ética e disciplinar.

Lisboa 7 de Outubro de 2019